Identification of the Brominated Flame Retardant 1,2-Dibromo-4-(1,2-dibromoethyl)cyclohexane as an Androgen Agonist

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Received June 13, 2006

To investigate androgen receptor (AR) activation by exogenous compounds, we used a combination of experimental analysis and theoretical modeling to compare a set of brominated flame retardants (BFRs) to dihydrotestosterone (DHT) with regard to ligand docking, AR binding, and AR activation in human hepatocellular liver carcinoma cells, as well as interacting energy analysis. Modeling of receptor docking was found to be a useful first step in predicting the potential to translocate to the ligand pocket of the receptor, and the computed interaction energy was found to correlate with the observed binding affinity. Flexible alignment studies of the BFR compounds demonstrated that 1,2-dibromo-4-(1,2-dibromoethyl)-cyclohexane (BCH) closely overlap DHT. Combining the theoretical modeling with in vitro ligand-binding and receptor-activation assays, we show that BCH binds to and activates the human AR. The remaining BFRs did not successfully interact with the ligand pocket, were not able to replace a synthetic androgen from the receptor, and failed to activate the receptor.

Introduction

Many human health consequences have been attributed to the exposure to endocrine disrupting substances.[1] While numerous oestrogen agonists have been identified, and shown to interfere with reproduction, the presence of androgenic compounds has so far been inferred from studies of masculinisation of animals.[2,3] However, several compounds have been shown to possess low androgen receptor (AR[a]) binding affinity and to act as AR antagonists.[4−6] Several studies have also shown that oestrogenic compounds can act as antiandrogens.[7,8] While no xenobiotics have so far been identified as being strong AR agonists, two compounds, 2-tert-butylanthaquinone (2tBAQ) and benzoanthrone (BA), were recently suggested to possess weak AR agonistic properties.[5]

It has been shown that 50% of all males have benign prostate hyperplasia at the age of 50 and that the incidence of prostate cancer is increasing.[9] In addition, among humans, exposure to androgens such as dihydrotestosterone (DHT) is known to result in hyperplasia of the prostate.[10]

As the presence of androgenic compounds could contribute to the increased androgenic effects observed in nature, the focus has increasingly been on the screening for, and identification of, androgenic compounds. In an analysis of 253 compounds, Araki and co-workers identified two compounds that bound to the AR and that appeared to weakly activate AR at high concentrations.[5] Together with studies showing activation of an androgen regulated gene in fish,[11,12] these studies demonstrate that we can expect to uncover androgenic compounds that are emitted into the environment with the potential to cause reproductive disturbances.

In the present study, we combine theoretical modeling with in vitro experiments on a set of 10 brominated flame retardants (BFRs; Figure 1a) to determine their potential to interact with the human AR by binding to the ligand-binding pocket and to determine if this results in activation of the receptor. The compounds were selected using experimental statistical design in combination with a multivariate chemical characterization to cover the chemical domain of BFRs.[13]

Results

Ligand Docking. Molecular modeling techniques were used to determine the ligand docking of the BFRs and DHT in the human AR ligand binding domain. After initial structure minimization of the receptor, the two main accessible sites for ligand interaction were identified (Figure 2a,b), located in the active site and on the receptor surface at Arg88/Gln46, respectively. The active site pocket of AR contains two hydrogen-bonding ends with Arg88, Gln 46 at one and Asn 41, Thr211 at the other, connected through a hydrophobic central funnel.[14] Flexible ligand docking of DHT verified the location of the active site, with DHT perfectly interacting with the four main H-bonding/polar amino acids Arg88, Gln46, Asn41, and Thr211.

All 10 BFRs were structure minimized, followed by automated flexible docking. Of the 10 BFRs, only 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (BCH; compound 10) and 2,4,4′-tribromodiphenylether (BDE28; compound 5) were able to dock well into the active site. The remaining compounds were all found docking to the entrance channel (Figure 2b) or were too small to be able to interact properly with both polar ends of the active site. Compounds 3, 6, and 9 are small enough to fit into the active site, where they mainly interact with the Asn and Thr groups, but were not able to reach across the active site to Arg88. Furthermore, DHT and BCH show clear overlap after docking and subsequent minimization.

In Vitro Ligand-Binding Assay. In a receptor binding-assay experiment, all 10 BFRs were tested using the PolarScreen AR

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[a] Abbreviations: AR, androgen receptor; BA, benzoanthrone; BCH, 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane; BDE28, 2,4,4′-tribromodiphenylether; BFR, brominated flame retardant; DHT, dihydrotestosterone; LIE, linear interaction energy; HepG2, human hepatocellular liver carcinoma cells; 2tBAQ, 2-tertbutylanthraquinone; 3BrP, 2,4,6-tribromophenol; 33DCB, 3,3′-dichlorobenzidine dihydrochloride; 4DMAB, 4-diethylaminobenzaldehyde; 6BrB, hexabromobenzene.

10.1021/jm060713d CCC: $33.50
Published on Web 11/17/2006

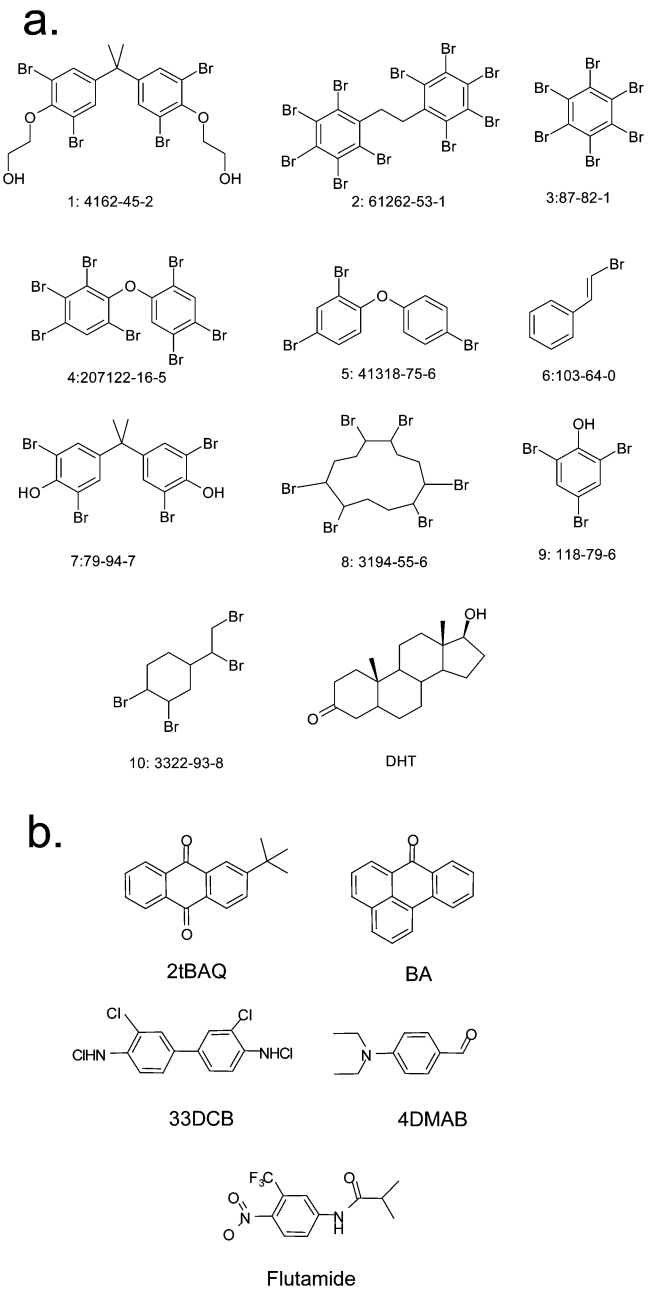

Figure 1. (a) Test set of BRFs explored in the current work, including their CAS registry numbers. Compounds 1 (tetrabrombisphenol A 2-hydroxyethyl ether), 2 (2,2′,3,3′,4,4′,5,5′,6,6′-decabromodipheylether), 3 (6BrB), 4 (2,2′,.3,4,4′,5′,6-heptabromodiphenylether), 5 (2,4,4′-tribromodipheylether, BDE28), 6 (β-bromostyrene), 7 (3,3′,5,5′-tetrabromobisphenol A), 8 (1,2,5,6,9,10-hexabromocyclododecane), 9 (2,4,6-tribromophenol, 3BrP), 10 (BCH). DHT is also included for comparison. (b) Four previously reported agonists/antagonists 2tBAQ, BA, ′33DCB 4DMAB, and flutamide.

competitive assay (PanVera) at 100 μM concentration. Only BCH was capable of replacing flourmone in the ligand-binding assay at this high concentration (data not shown). This suggests that only BCH will be localized to the ligand pocket of the AR. BCH was thereafter tested using concentrations ranging from 1 nM to 10 μM to determine its relative binding affinity. BCH was found to bind tightly to the receptor (Figure 3), with an IC_{50} of 163 nM, only 1 magnitude higher than DHT (18.5 nM). These experimental data are in agreement with the theoretical modeling showing that BCH has the best fit of the BFR into the ligand pocket.

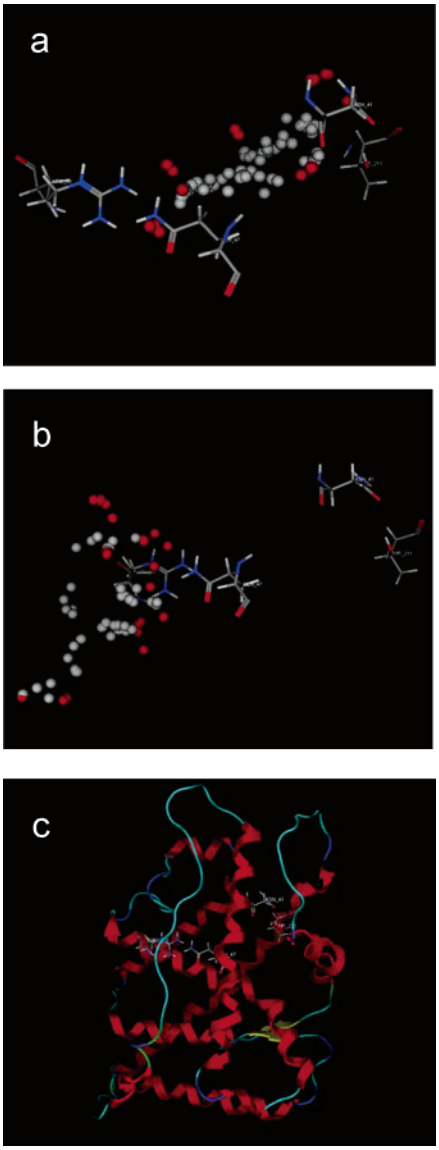

Figure 2. Human AR after energy minimization. (a) Main accessible region in the active site (white spheres, hydrophobic; red spheres, hydrophilic). (b) Main accessible region on the surface outside the Arg88/Gln46 groups. (c) Minimized structure of AR with the four key amino acids in active site shown in stick-model.

In Vitro AR Activation Assay. Using human hepatocellular liver carcinoma cells (HepG2), transiently transfected with the human AR and a luciferase reporter gene, we identified BCH as a strong activator of the AR (Figure 4). Of the tested compounds, only BCH was able to activate the human AR. To confirm the identity of the tested active compound (BCH) it was submitted to GC-MS analysis using an Agilent 5973 MSD instrument in EI-mode. The produced mass spectra were in agreement with the compound having the CAS# 3322-93-8 in the NIST98 mass spectra library. Thus, the identity of compound 10 (BCH) was confirmed. The remaining compounds did not activate AR or function as antagonists in coexposure assays. To determine the relative potency of BCH, a dose response experiment was performed where BCH was compared to DHT (Figure 5a). DHT reached a maximum 12-fold induction at 10^{-8} M with an IC_{50} of 1.82×10^{-9} M. BCH on the other hand reached maximum levels (3-fold induction) first at 10^{-5} M and had an IC_{50} of 4.27×10^{-8} M. Thus, the androgenic activity of DHT is 25-fold higher than that of BCH. While the ligand-binding affinity of BCH is comparable to that of testosterone

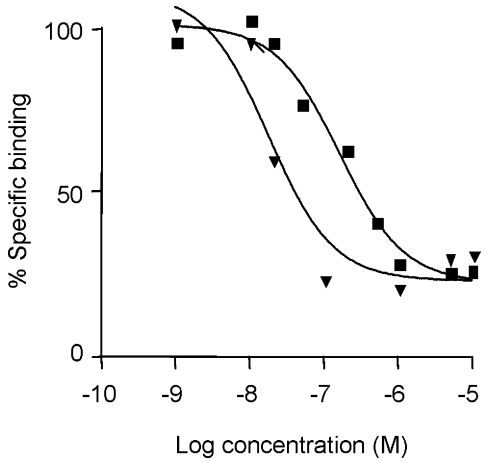

Figure 3. Competition curves for DHT (triangle) and BCH (squares) to the rat AR. The amino acid sequence of the ligand-binding domain of rat and human AR (GeneBank accession numbers J05454 and M20132, respectively) are identical, wherefore the results can be extrapolated to the human AR.

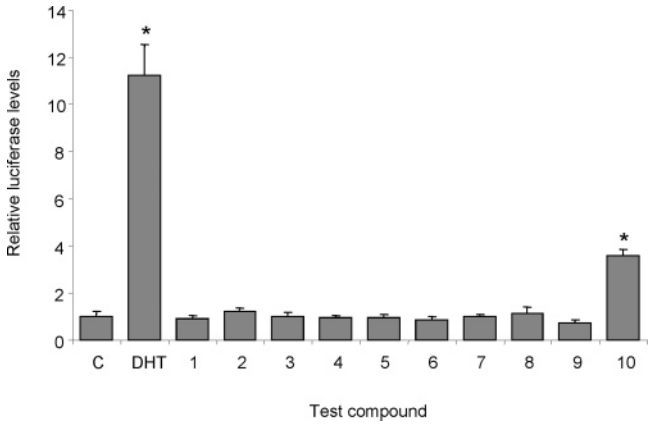

Figure 4. Luciferase reporter gene activation, following exposure of HepG2 to DHT at 10 nM and the different test compounds at 1 μM concentration. Each experiment was performed with $n = 4$. Statistically significant difference from the control level ($p < 0.01$) is indicated by an asterisk.

and 11-ketotestosterone, the activation potential is 1 magnitude lower than DHT, testosterone, or 11-ketotestoterone, which are equally potent at inducing the human AR.[15]

Exposure of the HepG2 to BCH in the presence of 100-fold excess of flutamide, a known androgen antagonist, resulted in a 60% reduction in luciferase signal (Figure 5b). Coexposure of HepG2 to 1 nM DHT together with 1 μM of each BFR showed that coexposure of DHT and BCH resulted in an additive response (Figure 5b), while the other BFR did not modulate the DHT response (data not shown). This indicates that BCH is a potent androgen agonist and suggests that low-level exposure to this compound results in androgenic effects and potential endocrine disruption.

Modeling of Ligand−Receptor Interactions. The main interactions during the MD simulations show that DHT remains hydrogen bonded by the hydroxyl group to the backbone amine group of Thr211 on one side and by the keto group to the Gln46 and Arg88 amine side chains on the other. For BCH, two possible orientations were found with very similar docking scores, differing in orientation through a 180-degree rotation. One bromine interacts mainly with the Arg88 amine group, whereas the remaining bromines primarily exhibit van der Waals interactions with polar methylene hydrogens in leucines and methionines in the ligand-binding pocket. These interactions

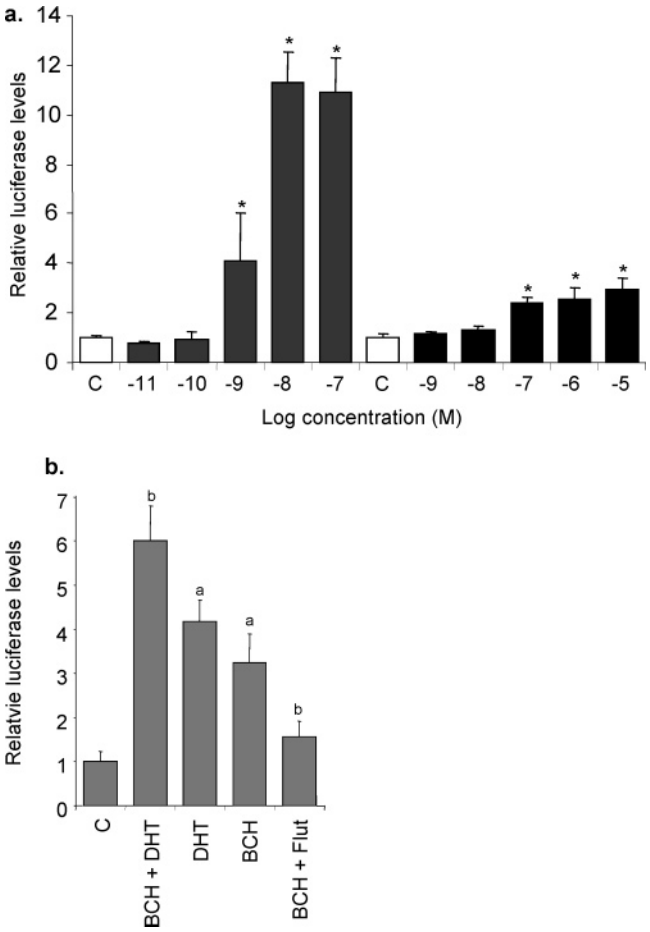

Figure 5. Interaction of AR with DHT and BCH. (a) Dose−response analysis of AR activation by DHT (gray bars) and BCH (black bars) compared to control (C) unexposed cells (white bars). HepG2 were transfected with human AR and ARE-*slp*-luc together and the Renilla reporter vector. The cells were treated with both substances in concentrations ranging from 1 nM to 10 μM. The results are shown as mean ± SD ($n = 4$). Statistically significant difference from the control level ($p < 0.01$) is indicated by an asterisk. (b) Coexposure of HepG2 to 1 nM DHT, 1 μM BCH alone, or in combination and coexposure of 1 μM BCH with 100 μM flutamide. Controls (C) were transfected with both constructs but not treated with any inducer. The results are shown as mean ± SD ($n = 4$). Statistically significant differences from the control level ($p < 0.01$) are indicated by an "a" and statistically significant differences from the induced DHT or BCH are indicated by a "b".

efficiently stabilize the structure to closely overlap with that of DHT (Figure 6a). The structural overlap between DHT and BCH was verified in flexible alignment studies, elucidating the structural and functional resemblance between these compounds relative to DHT and the remaining BFRs (Figure 6b).

The structures and Gauss−Conolly electrostatic surfaces of DHT, BCH, and BDE28 were compared, and we noted that, whereas DHT and BCH have very similar distances between the main polar groups, compound BDE28 is too long to fit ideally into the pocket and that the distorted structure due to the bridging oxygen and perpendicular orientation of the aromatic rings leads to relatively large structural rearrangements of the active-site side chains. In BDE28, the polar bridging oxygen will furthermore reside in the hydrophobic central region of the active site pocket.

The interaction energies between the ligand and the AR were computed using structures subjected to docking, relaxation, MD-simulation, and additional relaxation (Table 1). The MD simulations were essential to allow for the active site to properly

Table 1. Interaction Energies (kcal/mol) between a Set of Ligands and AR[a]

compound	interaction site	ΔE	compound	interaction site	ΔE
DHT	active site	48.8 (13.2)	BDE28 (5)	active site	27.1 (15.8)
flutamide	active site	48.7 (18.5)	6BrB (3)	active site	28.0 (12.0)
flutamide	active site (180°)	47.9 (15.5)	3BrP (9)	active site	27.3 (11.1)
flutamide	outside	50.8 (19.1)	2tBAQ	outside	27.9 (1.3)
BCH	active site	35.8 (14.8)	BA	active site	31.5 (12.5)
BCH	active site (180°)	27.9 (9.6)	33DCB	outside	32.7 (10.3)
BCH	outside	18.2 (−0.4)	4DMAB	active site	31.9 (6.5)

[a] "Outside" denotes the surface outside the Arg88, Gln46 group. Values in parenthesis are from a simplified LIE analysis.

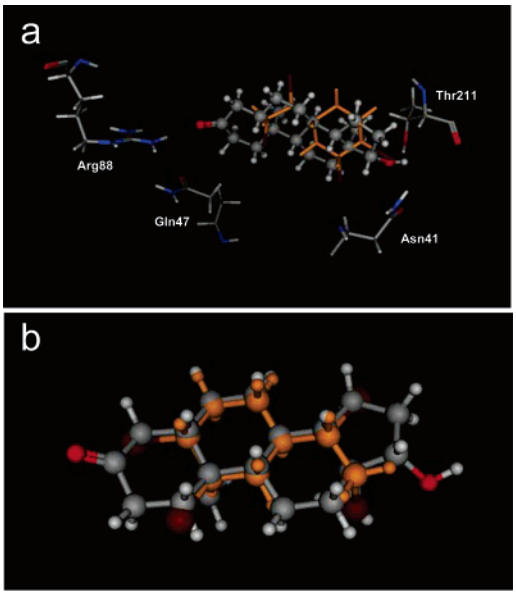

Figure 6. Alignment of DHT and BCH. (a) Close-up of the docked structures of DHT and BCH with best scores (prior to minimization and molecular dynamics simulations) in the human AR. DHT in atom-colored, ball-and-stick BCH in golden stick-model. Active site residues shown in atom-colored stick-model. (b) DHT (atom-colored) and BCH (golden) after flexible alignment.

account for ligand-induced structural modifications, as also noted in a recent multidimensional QSAR study of ligand binding to the AR receptor active site.[16] In addition to DHT and BFRs 3, 5, 9, and 10 (Figure 1a), we also investigated the interaction energies of flutamide and four compounds noted in the previous study by Araki and co-workers[5] to have weak androgenic (two compounds) or antiandrogenic (two out of nine compounds) activity (Figure 1b). Besides the "direct" interaction energy between ligand and receptor, a simplified linear interaction energy (LIE)[17] protocol, defined as the difference between the (van der Waals and electrostatic) interaction energies of the ligand with the receptor and with water, were evaluated for all the above systems.

DHT, being a strong activator of the receptor, has an interaction energy with the receptor of 49 kcal/mol, highly similar to flutamide which lies in the range 47−51 kcal/mol. It should be noted that, for flutamide, both orientations inside the active site (180 degree rotated) and the substrate docked on the surface outside the Arg88, Gln 46 groups all display very similar interaction with AR, which may account for its strong competing capacity with DHT. BCH attains interaction values 28 and 36 kcal/mol for the two orientations inside the active site pocket, respectively, whereas testing the interaction at the outside entrance channel with best docking score gave an interaction energy of only 18 kcal/mol (also associated with large movements of the ligand away from the docked position during the MD simulation).

The two small BFRs, hexabromo benzene (compound 3) and 2,4,6-tribromo phenol (compound 9), both display interaction energies with the AR active site of 27−28 kcal/mol, that is, similar to one of the BCH orientations. The two compounds claimed to show weak androgenic activity, 2tBAQ and BA, have different optimum docking sites. 2tBAQ preferentially resides at the outside-lying docking site, whereas BA attained its highest docking score inside the active site pocket. The interactions energy of 2tBAQ is of the same order as seen for the inactive BFR compounds, whereas BA is somewhat higher, 32 kcal/mol, yet still 4 kcal/mol lower than BCH. Similarly, two compounds 3,3′-dichlorobenzidine dihydrochloride (33DCB) and 4-diethylaminobenzaldehyde (4DMAB) were selected for detailed MD-interaction studies out of the nine compounds reported to have weak antiandrogenic activity.[5] The selections were based on the fact that these molecules have two polar ends and are of similar size as DHT and BCH. Initial docking was, however, performed on all nine substances. Both 33BCD that docks on the outside and 4DMAB that docks inside the active-site pocket display similar interaction energies as the weakly androgenic compound BA. For all compounds, the dominant term is the van der Waals energy (25−40 kcal/mol), whereas the electrostatics comprise a far smaller part of the total interaction (0−15 kcal/mol). The largest electrostatic interaction is seen for DHT and flutamide (∼15 kcal/mol), whereas for BCH, BDE28, and hexabromobenzene (6BrB), the electrostatic contribution is close to zero.

Data from the simplified LIE analysis is also included in Table 1. The strongest LIE is displayed by flutamide, whereas DHT, BCH, and BDE28 all display very similar LIE values (∼15 kcal/mol). The LIE data for 6BrB, 3BrP, BA, and 33DCB, in turn, are a couple of kcal/mol lower than these. It should be emphasized, however, that the LIE analysis performed herein is highly limited and thus associated with large uncertainty.

From the modeling studies, we propose that a ligand−receptor interaction energy below approximately 30 kcal/mol (within the current methodology) is not sufficient for altering the receptor activity and that interactions in the range 31−33 kcal/mol (as displayed by, e.g., BA, 33DCB and 4DMAB) give weak alterations of activity, whereas interactions above 35 kcal/mol (BCH) can result in relatively strong receptor activation. We furthermore note that careful analyses are required to validate whether a compound will activate the AR or not and that the modeling studies do not reveal whether the interacting compound is an agonist (androgenic) or antagonist (antiandrogenic). In addition, the similar interaction energy and LIE values of BCH (agonist) and BDE28 (no binding to or activation of AR) clearly illustrate the fact that the different behavior displayed in vitro by these compounds cannot be fully assessed by current modeling. For example, the capability to diffuse into the active-site pocket is not addressed, and subsequent activation mechanisms of the AR + ligand complex are not within reach of

current-day modeling. The modeling provides an efficient tool to screen compounds and to highlight key features of potential receptor binding ligands, but will inherently be associated with the capacity to generate "false positives", as in the case of BDE28.

Discussion

The present study shows that modeling is a powerful tool for an initial screening to determine if compounds will translocate to the ligand-binding pocket and to determine the interaction energies. However, experimental analysis remains essential for the determination of agonist and antagonist properties of identified compounds and to rule out false positives from the modeling results. Using the above approach, the combined results show that BCH is a strong AR agonist with a potential of causing endocrine disruption, even at relatively low exposures. Information on the production and release of BCH into the environment is lacking, but the reported production levels in 2002 were between 10 000 and 500 000 pounds.[18] As the compound is used as an additive in construction material, high-impact plastic parts of appliances, and electric cable coating, it is reasonable to expect a substantial leakage into the environment of this and similar compounds. In the present study, we have used three complementing techniques to assess the androgenicity of BCH. Using receptor activation and coexposure to a known androgen (DHT) and antiandrogen (flutamide), we conclude that BCH activate the AR system in a reporter assay. Furthermore, we show that the BCH can compete with DHT in a receptor-binding assay. In addition, we determined the feasibility for the compounds to dock and interact with all four polar amino acids as well as the hydrophobic central region in the ligand-binding pocket. These studies confirmed that BCH was the only tested compound that would preferentially be located in the ligand pocket. One additional compound, BDE28, was also theoretically able to dock into the ligand-binding pocket. However, the lack of competition of BDE28 with flourmone indicates that it does not access the ligand-binding pocket.

It was recently indicated that two other compounds had weak androgenic activity, and nine compounds showed weak androgen antagonistic properties.[5] We, therefore, performed modeling on all of these compounds to determine if they too would interact with the ligand-binding pocket. Only one of the suggested agonists, BA, localized to the pocket. But as it is a nonpolar compound in the molecular extension toward the key amino acids, it is not able to interact as strongly with the necessary amino acids as, for example, BCH. The second compound, 2tBAQ, was only able to dock on the outside of the receptor. It is, therefore, unlikely that these two compounds are true androgen agonist, interacting with AR through the ligand-binding pocket. This may explain the low binding affinity and minimal activation obtained with these compounds. Of the nine antagonists, it was suggested that only four of them bound to AR and that the others could interact with coactivators and thereby inhibit AR activation.[5] However, in our modeling experiment, only one of the four compounds proposed to bind to the ligand-binding pocket would actually fit into the active site. In addition, it was too small to interact with all key amino acids. Similar results were obtained for the five compounds that did not compete in the binding assay. This suggests that a combination of receptor activation assays, receptor binding assays, and molecular modeling provides a powerful tool to assess and ascertain the nature of the interaction between ligands and receptors. Using this approach, we have identified the first pollutant that interacts with the AR and results in substantial activation of the receptor. As androgenic effects have been identified in the environment, the present results give an indication that there may be several hitherto unidentified androgenic endocrine disruptors remaining to be identified.

Conclusions

In the present study, we have for the first time identified an environmental chemical with properties that render it a strong AR agonist. Using the information obtained for BCH together with information on weak AR agonists, we show that molecular modeling is a powerful tool for the initial screening of compounds with possible endocrine disrupting effects. An approximate threshold level for interaction energy required for a compound to act as either agonist or antagonist can be deduced from the present study. It is concluded that docking and energy minimization alone is not sufficient to single out active compounds; instead, MD-assisted induced fit and subsequent energy minimization is necessary to allow for the receptor to maximize its interactions with the ligand. In addition, we postulate a molecule should be 10−11 Å long and have polar/polarizable/H-bonding ends and a nonpolar/hydrophobic central region to provide AR activity. Failure to fulfill any of these three criteria appears to render significantly reduced activity. However, it remains that the experimental analysis of the reporter system activity is needed to determine if a compound that based on modeling data lies within the interaction energy threshold, it will in fact reach the active site pocket and subsequently act as an agonist or an antagonist.

Materials and Methods

Chemicals. The three PBDEs (compounds 2, 4, and 5) were synthesized at Environmental Chemistry, Stockholm University. Compounds 1, 3, 6, 8, 9, and 10 were purchased from Sigma-Aldrich (Stockholm, Sweden). Compound 7 was purchased from Promochem (Borås, Sweden). The CAS registry numbers for the studied compounds are given in Figure 1.

Computational Modeling. The crystal structure of the human AR ligand binding domain (pdb entry 1e3g) was energy minimized with an energy gradient of 0.05 using the Amber99 force field by Kollman and co-workers,[19,20] employing the molecular operating environment (MOE) program.[21] The compounds listed in Figure 1 were initially constructed and energy minimized with the MMFF94X force field,[22−26] specially designed for small organic compounds. Flexible alignment[27] of the different ligands toward DHT was performed, searching for 100 possible aligned structures. The scoring was evaluated based on the lowest *S*-value (the objective function, based on evaluating the affinity ΔG scoring function; a combination of strain energy and mutual similarity score).

Monte Carlo simulated annealing based flexible docking of the ligands into the AR was performed using automated docking as incorporated in MOE, with a maximum of 500 docking conformations evaluated for each system. Again, the docked structures were sorted according to lowest objective function. The alpha site finder protocol[28] within the MOE program was employed to identify the accessible docking sites. Two sites stood out as being the dominant ones; one representing the active site and the other on the surface outside the Arg/Gln groups. In the docking simulations, all ligands were furthermore found to dock to either of these two sites only, again illustrating the dominant character of these. The lowest structures for each system were further energy minimized together with the entire receptor, and subjected to molecular dynamics simulation to collect better data on specific interactions in the active site. For the MD simulations, a NVT ensemble was used, and the systems were subjected to 20 ps heating and equilibration from 100 to 300 K, followed by 1 ns simulations at 300 K with 2 fs time steps. The resulting MD structures were energy minimized

again, and the interaction energy was evaluated by comparing the energy of the minimized complexes to those of the isolated, minimized reactants.

A simplified LIE[17] approach was also employed in which the interaction energy of the ligand with water was compared with the interaction energy between ligand and receptor. Water interaction was obtained by embedding the ligand in a solvent shell with radius 10 Å (a total of 250−350 water molecules surrounding each ligand), followed by a short MD simulation and energy minimization using the Amber99 force field. In the aqueous environment and when interacting with the receptor, the conformations of the ligand and the surroundings were held static (not allowed to relax) when determining the interaction energies.

In all force field calculations and simulations, the systems were surrounded by a dielectric medium with $\epsilon = 80$, modeled using a modified generalized Born model, as implemented in the MOE package.[21,29]

Receptor Binding Assay. Competitive binding assays for AR were performed using the PolarScreen AR competitor assay according to the PanVera protocol, using polarization fluorometry analysis, with excitation at 485 nm and emission at 535 nm, on GeniosPro instrument (Tecan Trading AG, Switzerland). The final concentration of AR-LBD was 50 nM, and the androgen fluormone was 2 nM. DHT was used as a positive control. All BFR were tested for interactions at 100 μM concentration. Competition curves were produced for DHT and BCH using concentrations ranging from 1 nM to 10 μM. All tests were performed in duplicate.

AR Activation Assay. To test receptor activity, a HepG2 line was used. Cells were cultured in E-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Biochrom AG, Berlin, Germany), and antibiotic antimycotic (Invitrogen) in an incubator at a stable environment of 80% humidity, 5% CO_2, and 37 °C. Prior to transfection, the cells were transferred to antibiotics-free medium and, thereafter, seeded onto 24-well plates. At 90−95% confluence, the cells were transfected with 0.6 μg DNA (270 ng ARE-*slp*-Luc reporter, 270 ng human AR expression vector (pCMVhAR), and 60 ng pRL (Promega, Madison) using lipofectamine 2000 (Invitrogen) according to the manufacturers recommendations.

At 24 h post-transfection, the media was replaced with phenol-free media complemented with charcoal-stripped FCS containing DHT, different BFR, DHT + BCH, or BCH + flutamide. Cells were exposed for 40 h, where after the luciferase levels were measured using a Dual Luciferase Assay Kit (Promega) in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). The luciferase value received at each assay was normalized to the corresponding *Renilla* luciferase value.

Acknowledgment. This work was supported by grants from the Swedish Research Council, Swedish Animal Welfare Agency, The Royal Swedish Academy of Science and Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning, and the Swedish research programme New Strategies for Risk Management of Chemicals. We would like to thank Dr. Elisabeth Wilson, University of North Carolina, for the kind gift of the human AR expression vector (pCMVhAR) and Dr. Guy Verrijdt, University of Leuven, Belgium, for the kind gift of the ARE-luciferase vector (ARU-*slp*-Luc). We would also like to thank Professor Åke Bergman and Ulrika Örn, Environmental Chemistry, Stockholm University, Sweden, for their kind gift of brominated diphenylethers.

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JM060713D